REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
2008	Open Literature	

4. TITLE AND SUBTITLE

Gas chromotographic-mass spectrometric analysis of sulfur mustard-plasma protein adducts: Validation and use of a rat inhalation model

5a. CONTRACT NUMBER

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Capacio, BR, Smith, JR, Lawrence, RJ, Boyd, BL, Witriol, AM, Conti, ML, Collins, JL, Sciuto, AM

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Chemical Defense
ATTN: MCMR-CDT-D
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

8. PERFORMING ORGANIZATION REPORT NUMBER

USAMRICD-P07-039

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

US Army Medical Research Institute of Institute of Chemical Defense
ATTN: MCMR-CDZ-P
3100 Ricketts Point Road
Aberdeen Proving Ground, MD 21010-5400

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for public release; distribution unlimited

13. SUPPLEMENTARY NOTES

Published in special issue of Journal of Analytical Toxicology, 32, 37-43, 2008

14. ABSTRACT

See reprint.

15. SUBJECT TERMS

Sulur mustard, plasma protein adducts, diagnostics, chemical agent, inhalation model

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
					Benedict R. Capacio
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**	UNLIMITED	7	**19b. TELEPHONE NUMBER** *(include area code)*
UNLIMITED	UNLIMITED	UNLIMITED			410-436-1944

Gas Chromatographic–Mass Spectrometric Analysis of Sulfur Mustard-Plasma Protein Adducts: Validation and Use in a Rat Inhalation Model*

Benedict R. Capacio†, J. Richard Smith, Richard J. Lawrence, Brian L. Boyd, Alicia M. Witriol, Michele L. Conti, Jennifer L. Collins, and **Alfred M. Sciuto**

Analytical Toxicology Division, U.S. Army Medical Research Institute of Chemical Defense, 3100 Ricketts Point Road, Aberdeen Proving Ground, Maryland 21010-5400

Abstract

Sulfur mustard (HD) is an alkylating agent that reacts rapidly with macromolecular targets resulting in the formation of stable adducts providing depots for markers of exposure. The purpose of this study was to validate an analytical procedure for detection of HD-plasma protein adducts and to establish the utility of the method in an HD rat inhalation study. Calibration curves were prepared in human and rat plasma at six levels of HD (12.5 to 400nM). Correlation coefficients for the mean data were 0.9987 for human and 0.9992 for rat plasma. The percent coefficient of variation (%CV) derived from the mean concentration data ranged from 0.53 to 14.1% in human (n = 5) and 0.57 to 10.63% in rat (n = 6) plasma. Intraday and interday precision and accuracy studies were conducted at three concentration levels (25, 150, 300nM) to represent low, medium, and high concentrations of HD relative to those employed in the calibration curve. Precision and accuracy were assessed by determining %CV and % error, respectively. For intra- and interday studies, the %CVs and absolute % errors were less than 15%. The limits of quantitation were 20.88nM for human and 16.73nM for rat plasma. In animal studies, rats received nebulized HD at six doses. The data indicate a dose-dependent relationship between maximal plasma concentrations and dose administered (R^2 = 0.9728). Results from this study indicate an accurate, precise, and sensitive method. The method was useful in determining plasma protein adduct formation in a rat inhalation model.

Introduction

Exposure to vesicating agents such as 2,2'-dichlorodiethyl sulfide (sulfur mustard; HD) is a concern of both civilian and military populations. Sulfur mustard is an alkylating agent that reacts rapidly with water, peptides, proteins, ribonucleic acid, and deoxyribonucleic acid (1). Reactions with macromolecular targets can result in the formation of stable adducts, which provide depots for markers of exposure (2–4). Theoretically, macromolecular markers may be present until eliminated by metabolic processes. These sources can potentially be leveraged as methods to verify exposure to the agent at relatively long periods following the event (2–4). Reviews on assays designed to detect exposure to chemical warfare agents involving free metabolites as well as macromolecular adducts have been published (2–5). For the latter group, assays include the immunoassay of HD bound to DNA (6), the gas chromatography–mass spectrometry (GC–MS) assay for the N-terminal valine adduct of hemoglobin (7–10), a GC–MS assay of thiodiglycol (TDG) from skin keratin (9), the liquid chromatography–MS–MS assay of the cysteine adduct in albumin (11), and an immunological method for the detection of mustard adducts to skin tissue (12).

The studies in this current report describe validation and further use in an HD inhalation study of a previously reported method for detecting HD exposure (13). The technique leverages interaction of HD with plasma proteins to form stable adducts. The analytical methodology involves isolation of plasma proteins, base catalyzed cleavage of the adduct followed by chemical derivatization and GC–MS assay of the derivative. Similar to skin keratin, plasma proteins contain numerous free carboxylic acid groups from aspartic acid and glutamic acid that can be alkylated by the electrophilic HD to give hydroxyethylthioethyl (HETE) esters (Equation 1). These interactions provide a relatively high quantity of plasma protein-HD adducts when compared with adducts formed at the four terminal valine sites in globin or the single cysteine site in albumin (2).

The interaction of HD with proteins and the subsequent steps in the analytical method are based on the key reactions shown in Scheme 1. Formation of the protein-HETE ester that occurs in vivo is described in Equation 1. These esters can

* The views of the authors do not purport to reflect the position or policies of the U.S. Army or the Department of Defense.

† Author to whom correspondence should be addressed: Commander, U.S. Army Medical Research Institute of Chemical Defense, ATTN: MCMR-CDT-D/Dr. B.R. Capacio, 3100 Ricketts Point Rd., Aberdeen Proving Ground, MD 21010-5400. E-mail: benedict.capacio@us.army.mil.

Equation 1

$$\underset{\text{(protein)}}{\text{Protein-COO}^-} + \underset{\text{(HD)}}{\text{S(CH}_2\text{CH}_2\text{Cl)}_2} + \text{H}_2\text{O} \rightarrow \underset{\text{(protein-HETE ester)}}{\text{Protein-COOCH}_2\text{CH}_2\text{SCH}_2\text{CH}_2\text{OH}} + \text{HCl} + \text{Cl}^-$$

Equation 2

$$\underset{\text{(protein-HETE ester)}}{\text{Protein-COOCH}_2\text{CH}_2\text{SCH}_2\text{CH}_2\text{OH}} + \text{NaOH} \rightarrow \underset{\text{(TDG)}}{\text{S(CH}_2\text{CH}_2\text{OH)}_2} + \text{Protein-COONa}$$

Equation 3

$$\underset{\text{(TDG)}}{\text{S(CH}_2\text{CH}_2\text{OH)}_2} + 2\ \text{C}_6\text{F}_5\text{COCl} \rightarrow \underset{\text{(TDGPFB)}}{\text{(C}_6\text{F}_5\text{COOCH}_2\text{CH}_2)_2\text{S}} + 2\ \text{HCl}$$

Scheme 1. Key reactions of HD with proteins.

be readily cleaved or hydrolyzed with dilute base to yield TDG (Equation 2). The TDG can be derivatized with pentafluorobenzoyl (PFB) chloride to give the bis(pentafluorobenzoyl) ester of thiodiglycol (2,2'-thiobisethanol dipentafluorobenzoate, TDGPFB) (Equation 3) and analyzed by a GC–MS negative-ion chemical ionization (NICI) in the selected ion monitoring mode (9,14,15).

Although this method has been utilized in animal studies and in an accidental human exposure to HD, it has not been fully characterized (13,16). The purpose of this study was to 1. characterize and validate the plasma protein adduct assay in plasma and 2. demonstrate the utility of the method in a procedure involving the inhaled administration of HD as a nebulized aerosol directly into the lung of a rat. Results from this study indicate an accurate, precise and sensitive method. The method was useful in determining plasma protein adduct formation in a dose-dependent fashion in a rat inhalation model. Herein, we report the details of the method validation and its use in vivo.

Experimental

Materials

Ethyl acetate, acetone, sodium bicarbonate, anhydrous sodium sulfate, pyridine, and PFB chloride were obtained from Sigma-Aldrich (St. Louis, MO) and used without purification. Sulfur mustard was obtained from the U.S. Army Edgewood Chemical Biological Center (Aberdeen Proving Ground, MD); purity as determined by nuclear magnetic resonance spectroscopy was 97.5%. The internal standard octadeuterothiodiglycol (TDG-d_8) was obtained from Ash Stevens (Detroit, MI). Bond Elut silica 100-mg Si solid-phase extraction (SPE) cartridges (Varian, Harbor City, CA) were conditioned with 1 mL of ethyl acetate before use. Pooled human and rat plasma derived from sodium heparin anticoagulated whole blood was obtained from Bioreclamation (Hicksville, NY).

Animals

Animals were maintained and used under a program accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International). The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (National Research Council, 1996) and the Animal Welfare Act of 1966 (P.L. 89-544), as amended in an AAALAC International accredited facility. Male Sprague-Dawley rats (240–270 g) were used for all inhalation experiments. Animals were anesthetized intramuscularly (im) with the co-administration of xylazine (10 mg/kg) and ketamine (90 mg/kg). Following anesthesia, rats were intubated with glass Pasture pipette endotracheal tubes that were trimmed to 7 cm in length and fire-polished and taped in place to the rostrum of the rat using surgical tape. Rats were then placed in a glovebox exposure chamber (Baker, Sanford, ME) that was under a slight negative pressure (–1.0 to –0.5 cm H_2O). The endotracheal tube was connected to a nebulizer system (Aeroneb, Aerogen, Dangan, Galway, Ireland), which were in series between the rat and the ventilator. Sulfur mustard was delivered by the ventilation action of a rodent ventilator (Harvard model 883) that was set to a respiratory rate and tidal volume of 60–100 breaths/min and 2.5 mL, respectively. The HD was then added to the nebulization mixture, which consisted of 25% ethanol and 75% saline. The nebulizer consistently delivered a particle size of < 3 µm at a rate of 0.5 mL/min as determined by an aerodynamic particle analyzer (TSI model 3321, Shoreview, MN). The aerosol stream was driven into the rat through a two-way non-rebreathing (Hans Rudolph, Kansas City, MO) valve. Exhaled HD exited the rat through the outflow port of the Rudolph valve, into a flask containing 0.5% bleach, and then through a charcoal filter prior to HEPA filtration. The entire exposure circuit was a closed system.

To verify HD vapor concentrations, samples were obtained using a 100-µL gas-tight syringe at the entrance of the glass endotracheal tube approximately 12–15 cm from the outflow of the nebulizer. The concentrations were determined using Minicams™ (O.I. Analytical, CMS Field Products Group, Birmingham, AL) chemical agent monitors. The sample was loaded into a 10-ft heated sample line that ran through the glovebox wall and was pulled directly onto a preconcentrator tube (100-mm length, 3-mm i.d., 15-mm bed of Tenax) within the Minicams. Separation was achieved with a DB-1 column (15-m, 0.32-mm i.d., 5-µm film thickness, J&W Scientific, Folsom, CA) with a helium flow rate of 40 mL/min. Detection was accomplished with a flame photometric detector. Samples were quantified against HD calibration curves generated from standards. The programmed cycle time between sample collection and analysis was 7 min, which allowed the analysis of two samples within the 10-min exposure time frame.

For this dose-response study, rats received a single exposure to six levels of HD aerosol at the following doses 25, 75, 175, 225, 275, and 300 µg HD/min for 10 min. The total dose delivered to the animals over 10 min was 250, 750, 1750, 2250, 2750, and 3000 µg HD. Animals were deeply anesthetized with the im co-administration of xylazine (10 mg/kg) and ketamine (90 mg/kg) and then euthanized via exsanguination at 0, 0.5, 1, 3, 6, and 24 h after exposure to obtain whole blood samples (4–6 animals per time point). Protein obtained from plasma (1.0 mL) was prepared as described in Sample preparation.

Sample preparation

To obtain plasma, whole blood was centrifuged at 2000 × *g* for 30 min at 5°C; the plasma supernatant was then removed for protein precipitation. Protein from plasma samples obtained from exposed animals or spiked standards was isolated and prepared as described by Capacio et al. (13) with modifications. Ten milliliters of acetone was added to 1 mL of plasma, which was then placed on a shaker for 10 min. Following shaking, the mixture was centrifuged at 4300 × *g* for 10 min at 5°C (Allegra 25R, Beckman Coulter, Palo Alto, CA), and the supernatant was discarded. A second wash was performed with the addition of 6–7 mL acetone to the packed pellet material, following which the samples were placed on a shaker for 10 min. The samples were again centrifuged (4300 × *g* for 10 min at 5°C) and the supernatant discarded. This second acetone wash was repeated for a total of three washes. The precipitated protein samples were placed in a desiccator and allowed to thoroughly dry at room temperature. Following drying, 200 µL of 1M NaOH was added to approximately 25 mg of protein. The mixture was heated to 70°C until the solid material dissolved (approximately 2 h). Then 20 µL of TDG-d_8 (300 ng/mL) was added and mixed thoroughly. Following the addition of 70 µL of 3M HCl, 1.0 mL ethyl acetate was added to the samples, and then they were vortex mixed for 10 min. The samples were then dried with the addition of 200 mg sodium sulfate, vortex mixed for 10 min, and centrifuged. An aliquot (500 µL) of the ethyl acetate supernatant was removed, and 200 mg of sodium sulfate was added for further drying. To this aliquot of ethyl acetate were added 15 µL of pyridine, 20 µL of PFB chloride, and 10 µL of methanol to form the PFB derivatives of TDG and TDG-d_8. The mixture was then dried over sodium sulfate (200 mg). Bond Elut silica 100 mg Si SPE cartridges were conditioned with 1 mL of ethyl acetate before use. A 200-µL portion of the reacted mixture (ethyl acetate containing the derivatized sample) was passed over the preconditioned cartridge. Finally, a 400-µL volume of clean ethyl acetate was added to the cartridge. The two fractions (200 and 400 µL) were combined for the GC–MS analysis.

Calibration curves

Calibration curves were prepared in human and rat plasma at six concentration levels of HD (12.5, 25, 50, 100, 200, and 400nM). Calibration curves prepared from human plasma were made on five separate days, whereas those from rat plasma were made on six different days. For all samples, sulfur mustard in saline (40,000nM, 100 µL) was spiked into plasma (9.9 mL) to give a final concentration of 400nM. This sample was serially diluted with blank plasma (containing no HD) to obtain the desired concentration levels. The plasma spiked with HD was then incubated for 2 h at 40°C. Protein precipitated from the calibration curve samples was processed as described in Sample preparation. The actual HD concentration calculated for each day was derived from its respective calibration curve. Across days, the mean concentration, standard deviation (SD), percent coefficient of variation [%CV; (SD/mean) × 100] and percent (%) error (percent difference between calculated and expected concentrations) were calculated.

Intraday and interday precision and accuracy studies

Intraday and interday variability were used to assess precision and accuracy of the method. For these studies, test unknown samples were made at three HD levels (25, 150, and 300nM) to represent low, medium, and high concentrations on the calibration curve. To prepare the samples, human plasma was spiked to a level of 400nM HD. This standard was serially diluted with plasma, resulting in HD standard concentrations of 0, 25, 50, 100, 200, and 400nM. Equal volumes of the 400 and 200nM standards were mixed to obtain the 300nM test unknown; similarly equal volumes of the 100 and 200nM were mixed to make the 150nM test unknown. For intraday studies, five sets (n = 5) of test unknown samples were prepared and analyzed together. Precision was assessed by expressing the %CV. Accuracy was expressed as % error. Interday test unknown samples were prepared as described except that the samples were prepared and analyzed on five different days. A calibration curve was prepared and analyzed prior to each (intra- and interday) study. All test unknown samples were quantified by using the mean values for these calibration curves. The 100nM standards from the test unknown preparation scheme described were utilized as check standards to monitor assay performance. Four check standards were analyzed during the intraday study. For the interday study, a total of nine check standards were run, one at the end of day 1 and thereafter at the beginning and end of each subsequent study day (days 2–5).

Instrumentation

GC–MS separations were performed on an Agilent 6890 GC interfaced to an Agilent 5973 mass-selective detector. The GC was fitted with a 30-m × 0.25-mm internal diameter DB-5MS bonded phase capillary column (0.25-µm film thickness, J&W Scientific). Helium was used as the carrier gas in the constant flow mode (1.2 mL/min). The oven temperature was held initially at 80°C for 1 min, programmed from 80 to 225°C at 30°C/min, and held at 225°C for 11 min. Splitless injections of 1-µL volume were made using an Agilent 7683 autosampler. The injection port temperature was set at 250°C, split vent delay at 1 min, and the transfer-line temperature at 280°C. Typical retention times were 13.3 min for TDG-d_8PFB and 13.5 min for TDGPFB.

The MS analysis was conducted using NICI with methane as the reagent gas. The resolution capability of the Agilent 5973 mass selective detector is specified as unit mass. The source and quadrupole temperatures were set at 150 and 120°C, respectively. The molecular ion and the M+1 isotope ion were monitored for the TDGPFB (*m/z* 510 and 511) and TDG-d_8PFB internal standard (*m/z* 518 and 519). Relative to monitoring a single ion, two ions were used to provide further confirmation of peak identity. The *m/z* 511 and *m/z* 519 were utilized strictly as qualifier ions. However, single ions for both analyte TDGPFB (*m/z* 510) and internal standard TDG-d_8PFB (*m/z* 518) were used for quantification. The dwell time for each ion was 100 ms, resulting in a total scan rate of 2.15 cycles/s. The electron multiplier voltage was set at +400 volts relative to the autotune setting.

Data analysis

Graphic representations, linear regression analyses, calculations for HD concentrations, and other descriptive statistics were accomplished with GraphPad Prism software (version 3.02, GraphPad Software, San Diego, CA) or Microsoft Excel 2000. Ions used for quantification purposes were *m/z* 518 and 519 for the internal standard (TDG-d_8PFB) and *m/z* 510 and 511 for the analyte (TDGPFB). The area under the curve (AUC) was determined for TDGPFB and TDG-d_8PFB and was expressed as an AUC ratio (TDGPFB/TDG-d_8PFB), which was then normalized by the mass of protein utilized for each sample (AUC ratio/mg protein). Calibration curves depicting the relationship between AUC ratio/mg protein as a function of HD plasma concentration (nM) were utilized to express data as HD plasma concentration (nM).

Results and Discussion

The assay reported in this study has been utilized previously in research animal models and in an accidental human exposure to confirm HD as the agent involved (13,16). The purpose of the study reported herein is 1. to characterize the performance of the assay in terms of variability, linearity, and sensitivity and 2. to use the method to confirm delivery of different doses of HD administered as an inhaled nebulized aerosol from the lung to the blood compartment in a rat exposure model.

Calibration curves

Calibration curves were prepared from commercially purchased human and rat plasma. The purpose was to examine the linearity and variability (as determined by %CV and % error) in the matrices from both species. Linear regression analysis for human plasma samples indicated that the range of correlation coefficients (R^2) was 0.9974–0.9998 for individual days with a value for the mean data of 0.9987. Similarly for rat plasma, the range for individual days was 0.9990 to 0.9999 with the value for the mean data of 0.9992. Calibration curves constructed from human and rat plasma data demonstrated consistency from day to day. The %CVs derived from the mean concentration data at levels 12.5, 25.0, 50.0, 100, 200, and 400nM were 14.1, 9.55, 1.71, 1.30, 2.11, and 0.53%, respectively, in human (n = 5) and 10.63, 7.55, 2.34, 6.19, 2.65, and 0.57%, respectively, in rat (n = 6) plasma (Tables I and II). The data indicate that the % errors were in the acceptable range in accordance with FDA guidance (i.e., less than 15%) (17), except for the 12.5nM level in human plasma, which gave a mean concentration of 8.33nM, resulting in an error of 33.3%. Data from the

Table I. Calibration Curves from Human Plasma on Individual Days*

	Sulfur Mustard Standard (Expected Concentration)					
	12.5 nM	25 nM	50 nM	100 nM	200 nM	400 nM
Day	Calculated (nM)					
1	6.48	24.4	50.0	107.4	213.3	391.7
2	8.39	26.6	51.2	109.0	203.5	395.9
3	8.25	21.2	51.9	109.1	206.2	394.8
4	9.67	27.2	50.1	106.0	201.8	397.5
5	8.88	24.2	51.6	106.6	206.7	394.9
Mean concentration (nM)	8.33	24.7	51.0	107.6	206.3	395.0
SD	1.17	2.36	0.87	1.39	4.37	2.11
%CV	14.1	9.55	1.71	1.30	2.11	0. 53
% Error	–33.3	–1.20	2.00	7.60	3.15	–1.25

* The calculated HD concentrations were derived from respective daily calibration curves. The % coefficient of variation (%CV) was calculated by (SD/mean) x 100 (SD = standard deviation). The % error was calculated by percent difference between calculated and expected concentrations.

Table II. Calibration Curves from Rat Plasma on Individual Days*

	Sulfur Mustard Standard (Expected Concentration)					
	12.5 nM	25 nM	50 nM	100 nM	200 nM	400 nM
Day	Calculated (nM)					
1	11.44	23.16	49.00	108.26	199.56	398.43
2	11.33	26.47	50.74	95.05	209.15	396.51
3	12.15	26.64	49.93	99.91	201.06	399.41
4	14.43	29.11	49.02	96.28	196.52	402.47
5	13.82	28.07	50.69	90.04	208.18	398.08
6	13.90	26.78	47.75	98.14	197.83	401.68
Mean concentration (nM)	12.85	26.71	49.52	97.95	202.05	399.43
SD	1.37	2.03	1.16	6.06	5.36	2.26
% CV	10.63	7.55	2.34	6.19	2.65	0. 57
% Error	2.77	6.82	–0.96	–2.05	1.03	–0.14

* The calculated HD concentrations were derived from respective daily calibration curves. The % coefficient of variation (%CV) was calculated by (SD/mean) x 100 (SD = standard deviation). The % error was calculated by percent difference between calculated and expected concentrations.

Table III. Precision and Accuracy for Intraday Test Samples in Human Plasma*

Expected Concentration (nM)	Mean (n = 5) Calculated Concentration (nM)	Precision		Accuracy
		SD	%CV	% Error
300.0	292.94	5.52	1.88	–2.35
150.0	153.59	21.18	13.79	2.39
25.0	22.03	1.29	5.86	–11.87
0.0	0	0	0	0

* Five (N = 5) sets of test samples (0, 25, 150, and 300nM) in human plasma were made at once and analyzed together. Precision was assessed by expressing the % coefficient of variation (%CV) calculated by (SD/mean) x 100 (SD = standard deviation). Accuracy was expressed as % error and was calculated by percent difference between calculated and expected concentrations.

calibration curves in human and rat plasma demonstrate that the method is reproducible across all concentration levels employed in the study as indicated by the %CVs, which were less than 15%. However, accuracy at the low level (12.5nM) in the human data is relatively large (33.3%). This is because the value is below the limit of quantitation (LOQ) (20.88nM) determination (see Limits of detection and quantitation).

Precision and accuracy

Studies to examine intra- and interday variability were assessed by calculating precision and accuracy values for test samples in human plasma. The concentration levels (25, 150, 300nM) used in the studies were chosen to represent low, medium and high concentrations of HD relative to those employed in the calibration curve. Data demonstrating precision and accuracy of intra- and interday test samples are shown in Tables III and IV, respectively. For intraday samples, %CVs were 5.86, 13.79, and 1.88 for concentrations 25, 150, and 300nM, respectively. The accuracy as reflected by % error was –11.87, 2.39, and –2.35% for 25, 150, and 300nM, respectively. The %CVs for the interday samples were 14.64, 2.66, and 3.95 and % errors were 4.73, 2.62, and 2.53% for 25, 150, and 300nM, respectively. These data indicate that the assay demonstrates precision and accuracy at the studied HD levels, which are acceptable as defined by FDA guidance (i.e., less than 15%) (17). To monitor the assay performance, check standards (100nM) were run during the course of the intra- and interday studies. The calculated concentrations for the check standards run during the intra- and interday studies are presented in Table V. The mean calculated concentration for check standards run during the intraday study was 92.28nM with a %CV of 4.96% and % error of –7.72%. The calculated concentrations for the check standards (100nM) run during the interday study are presented in Table V. The mean calculated concentration was 98.92nM with a %CV and % error of 4.52 and –4.18%, respectively.

Table IV. Precision and Accuracy for Interday Test Samples in Human Plasma*

Expected Concentration (nM)	Mean (n = 5) Calculated Concentration (nM)	Precision		Accuracy
		SD	%CV	% Error
300.0	307.60	12.14	3.95	2.53
150.0	153.93	4.10	2.66	2.62
25.0	26.18	3.83	14.64	4.73
0.0	0	0	0.00	0.00

* Five (N = 5) sets of test samples (0, 25, 150 and 300nM) in human plasma were made and analyzed daily for five days. Precision was assessed by expressing the % coefficient of variation (%CV) calculated by (SD/mean) × 100 (SD = standard deviation). Accuracy was expressed as % error and was calculated by percent difference between calculated and expected concentrations.

Table V. Check Standards*

Intraday		Interday	
Standard number	Calculated concentration (nM)	Study day	Calculated concentration (nM)
1	87.67	1	94.81
2	96.72	2	97.88
3	89.07	2	93.30
4	95.67	3	103.24
-	-	3	105.94
-	-	4	100.51
-	-	4	102.92
-	-	5	94.23
-	-	5	97.47
Mean	92.28		98.92
SD	4.57		4.47
% CV	4.96		4.52
% Error	–7.72		–4.18

* Check standards made at a level of 100nM were run to monitor assay performance during the course of intra- and interday studies. For the intraday study, four check standards were run during the course of the study day. For interday studies, the check standards were run at the end of day 1 and thereafter at the beginning and end of each subsequent study day (interday 2–5) for a total of nine check standards.

Limits of detection (LOD) and quantitation (LOQ)

The LOD and LOQ were determined by the relationship $3S_0$ and $10S_0$ respectively (18,19). The S_0 was calculated as presented (19) and derived from the y-intercept of plots of standard deviations from calibration curves as a function of the respective HD concentrations (12.5, 25, 50, 100, and 200nM). For these assessments, data from seven calibration curves run over a five-week period were utilized from human plasma. For rat studies, data from calibration curves run along with unknown samples from all the inhalation studies were utilized. The rat studies in total encompassed a period of 18 months. The analysis indicated that the LOD and LOQ for rat and human plasma were similar. The LOD was determined as 6.26nM for human and 5.02nM for rat, and LOQ was 20.88nM for human and 16.73nM for rat plasma. The LOQ determination in human plasma is consistent with the relatively large % error obtained from the lowest level (12.5nM, 33.3%) in the calibration curves from human plasma. That is, at that level the assay did not produce quantifiably valid results. For a similar assay, Capacio et al. (13) reported the lowest level on the calibration curve used to quantify samples as 25nM, because that was the lowest standard utilized on the calibration curve with a signal/noise ratio of ≥ 3:1. Although the LOQ determined in this study (20.88nM) was derived differently, it is similar to that reported by Capacio et al. (13). Recently, numerous modifications to the procedure described in this present study have been implemented (20). They have resulted in approximately a 10-fold increase in sensitivity (LOQ = 1.6nM) relative to that reported in this present study (20).

Rat inhalation studies

The utility of the method was demonstrated in a rat inhalation model using nebulized HD. The graphic representation of HD plasma concentration as a function of time for each dose is depicted in Figure 1. In this study, HD was detectable in the plasma of the exposed animals out to the last time point examined (24 h). The data indicate a dose-dependent relationship

between maximal HD plasma concentrations and dose administered. Mean maximal plasma HD concentrations were 33.61, 107.1, 335.6, 390.7, 566.0, and 513.5nM for doses 250, 750, 1750, 2250, 2750, and 3000 µg, respectively. Regression analysis of mean maximal plasma HD concentration as a function of dose produced an R^2 of 0.9728 (Figure 2). Although the regression analysis indicates a linear relationship between dose and maximal plasma concentration, the relationship does not appear to hold at the two highest doses with respect to the maximal plasma concentrations and the overall time-course. Potential explanations for the observation include animal to animal variability and/or saturation of processes in the lung that are involved in partitioning of HD into the circulation at these higher levels of exposure. More studies are necessary to further elucidate the dose-plasma concentration relationship at this level of HD administration.

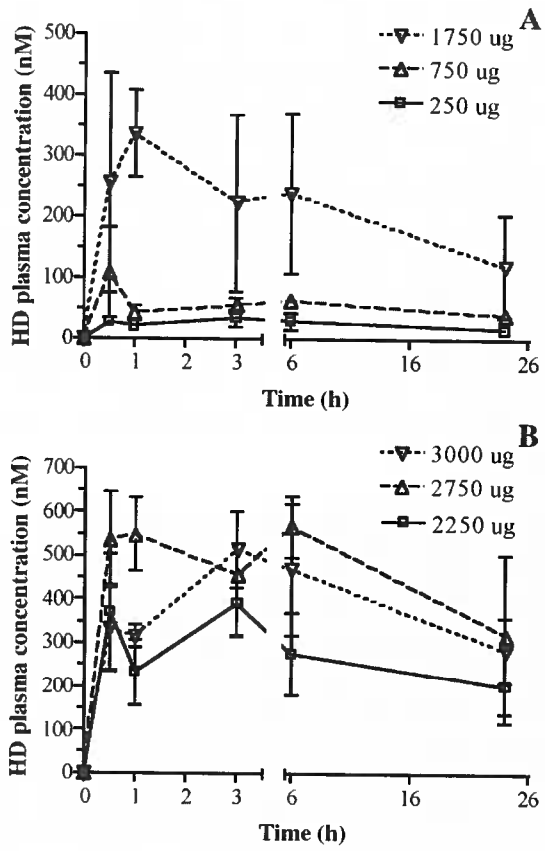

Figure 1. Plasma concentration-time profile of inhaled nebulized HD in rats. Animals received HD directly into the lung as a nebulized aerosol at six doses over 10 min: 2250, 2750, and 3000 µg HD (A) and 250, 750, and 1750 µg HD (B). Blood was obtained from 4–6 animals per time point at 0, 0.5, 1, 3, 6, and 24 h after exposure. Data are mean HD plasma concentration plus or minus the standard deviation.

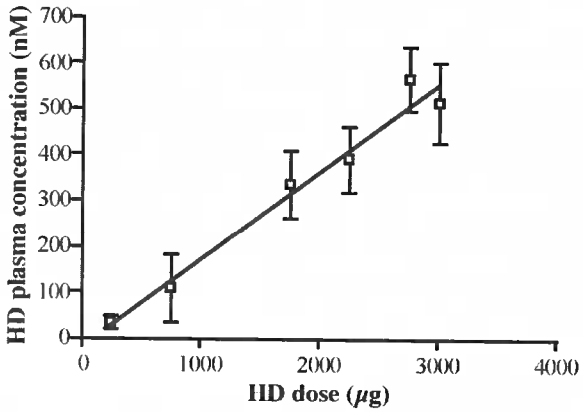

Figure 2. Maximal plasma concentration of HD and dose administered. Animals nebulized HD aerosol for total doses of 250, 750, 1750, 2250, 2750, and 3000 µg. The maximal HD plasma concentration obtained (± SD) as a function of dose delivered are plotted. The data indicate a linear relationship as reflected by a correlation coefficient of 0.9728.

This is the first study to examine the systemic presence of an HD biomarker from inhaled and nebulized HD driven directly into the lung by ventilatory action in the anesthetized rat. In other HD inhalation models (i.e., nose only administration) (21), many of the systemic effects may not be observed since detoxification processes take place in the nasal passages. This phenomenon can scrub a significant amount of an inhaled toxicant in obligate nose breathers (22), and data regarding the true effects of inhaled HD on lung toxicity and the role of the lung in systemic absorption processes may not be clear. In addition, the well-known effect of airway mucus plug formation can lead to ingestion and subsequent systemic HD poisoning via absorption from the gut. Models using whole-body vapor exposures of other chemical agents do not characterize systemic absorption exclusively through the lung, but represent a composite of numerous processes that contribute to total blood levels (23). As such, these models do not truly represent HD that enters the systemic circulation entirely via the lung. Advantages to the model utilized in the experiments reported herein are that it enables the study of HD that is directly focused on the lung and it minimizes interactions with other systems such as airway components or skin. Therefore, a direct relationship between dose delivered and blood levels and or toxicity can be obtained. The model will provide significant data regarding the true effects of inhaled HD and the role of the lung in systemic absorption, distribution, metabolism, and excretion processes.

Conclusions

A previously reported GC–MS method for determining in vivo exposure to HD has been validated. The method leverages the binding of HD to plasma proteins with subsequent cleavage and derivatization of the adduct and GC–MS-NICI analysis. Results from this study indicate an accurate, precise and sensitive method. Variability determined in precision and accuracy studies was less than 15%. The LOQs determined in human and rat plasma were calculated to be 20.88nM and 16.73nM, respectively. The use of the method in a rat inhalation model demonstrates the presence of the plasma protein biomarker in a dose-dependent fashion after the delivery of nebulized HD to the lung. Regression analysis of mean maximal plasma HD concentration as a function of dose produced an R^2 of 0.9728.

Acknowledgment

This research was supported by the Defense Threat Reduction Agency – Joint Science and Technology Office, Medical S&T Division.

References

1. P. Taylor. Anticholinesterase agents. In *Goodman and Gilman's The Pharmacological Basis of Therapeutics,* 11th ed., L.L. Brunton, J.S. Lazo, K.L. Parker, Eds. McGraw-Hill, New York, NY, 2006, pp 201–216.
2. D. Noort, H.P. Benschop, and R.M. Black. Biomonitoring of exposure to chemical warfare agents: a review. *Toxicol. Appl. Pharmacol.* **184:** 116–126 (2002).
3. D. Noort and R.M. Black. Methods for the retrospective detection of exposure to toxic scheduled chemicals. Part B: mass spectrometric and immunochemical analysis of covalent adducts to proteins and DNA. In *Chemical Weapons Convention Chemicals Analysis: Sample Collection, Preparation, and Analytical Methods,* M. Mesilaakso, Ed. John Wiley & Sons, Chichester, West Sussex, England, 2005, pp 433–451.
4. R.M. Black and D. Noort. Biological markers of exposure to chemical warfare agents. In *Chemical Warfare Agents Toxicology and Treatment,* 2nd ed. T.C. Marrs, R.L. Maynard, and F.R. Sidell, Eds. John Wiley & Sons Chichester, West Sussex, England, 2007, pp 127–156.
5. R.M. Black and D. Noort. Methods for the retrospective detection of exposure to toxic scheduled chemicals. Part A: analysis of free metabolites. In *Chemical Weapons Convention Chemicals Analysis: Sample Collection, Preparation, and Analytical Methods,* M. Mesilaakso, Ed. John Wiley & Sons, Chichester, West Sussex, England, 2005, pp 403–431.
6. A. Fidder, G.W. Moes, A.G. Scheffer, G.P. Van der Schans, R.A. Baan, L.P.A. de Jong, and H.P. Benschop. Synthesis, characterization and quantitation of the major adducts formed between sulfur mustard and DNA of calf thymus and human blood. *Chem. Res. Toxicol.* **7:** 199–204 (1994).
7. A. Fidder, D. Noort, A.L. de Jong, H.C. Trap, L.P. de Jong, and H.P. Benschop. Monitoring of in vitro and in vivo exposure to sulfur mustard by GC/MS determination of the N-terminal valine adduct in hemoglobin after a modified Edman degradation. *Chem. Res. Toxicol.* **9:** 788–792 (1996).
8. R.M. Black, R.J. Clarke, and J.M. Harrison. Biological fate of sulphur mustard: identification of valine and histidine adducts in haemoglobin from casualties of sulphur mustard poisoning. *Xenobiotica* **27:** 499–512 (1997).
9. D. Noort, A. Fidder, A.G. Hulst, L.P.A. de Jong, and H.P. Benschop. Diagnosis and dosimetry of exposure to sulfur mustard: development of a standard operating procedure for mass spectrometric analysis of haemoglobin adducts: exploratory research on albumin and keratin adducts. *J. Appl. Toxicol.* **20:** S187–S192 (2000).
10. D. Noort, A. Fidder, H.P. Benschop, L.P.A. de Jong, and J.R. Smith. Procedure for monitoring exposure to sulfur mustard based on modified Edman degradation of globin. *J. Anal. Toxicol.* **28:** 311–315 (2004).
11. D. Noort, A.G. Hulst, L.P.A. de Jong, and H.P. Benschop. Alkylation of human serum albumin by sulfur mustard in vitro and in vivo: mass spectrometric analysis of a cysteine adduct as a sensitive biomarker of exposure. *Chem. Res. Toxicol.* **12:** 715–721 (1999).
12. G.P. van der Schans, D. Noort, R.H. Mars-Groenendijk, A. Fidder, L.F. Chau, L.P.A. de Jong, and H.P. Benschop. Immunochemical detection of sulfur mustard adducts with keratins in the stratum corneum of human skin. *Chem. Res. Toxicol.* **15:** 21–25 (2002).
13. B.R. Capacio, J.R. Smith, M.T. DeLion, D.R. Anderson, J.S. Graham, G.E. Platoff, and W.D. Korte. Monitoring sulfur mustard exposure by gas chromatography–mass spectrometry analysis of thiodiglycol cleaved from blood proteins. *J. Anal. Toxicol.* **28:** 306–310 (2004).
14. R.M. Black and R.W. Read. Detection of trace levels of thiodiglycol in blood, plasma and urine using gas chromatography–electron-capture negative-ion chemical ionisation mass spectrometry. *J. Chromatogr.* **449:** 261–270 (1988).
15. R.M. Black and R.W. Read. Methods of the analysis of thiodiglycol sulphoxide, a metabolite of sulphur mustard, in urine using gas chromatography–mass spectrometry. *J. Chromatogr.* **558:** 393–404 (1991).
16. J. Newmark, J.M. Langer, B.R. Capacio, J.R. Barr, and R.G. McIntosh. Liquid sulfur mustard exposure. *Mil. Med.* **172:** 196–198 (2007).
17. Guidance for Industry, Bioanalytical Method Validation, U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), May 2001.
18. L.H. Keith, W. Crummett, J. Deegan, Jr., R.A. Libby, J.K. Taylor, and G. Wentler. Principles of environmental measurement. *Anal. Chem.* **55:** 2210–2218 (1983).
19. J.K. Taylor. Principles of measurement. In *Quality Assurance of Chemical Measurements.* Lewis Publishers, Chelsea, MI, 1990, pp 78–80.
20. R.J. Lawrence, J.R. Smith, B.L. Boyd, and B.R. Capacio. Improvements in the methodology of monitoring sulfur mustard exposure by gas chromatography–mass spectrometry analysis of cleaved and derivatized blood protein adducts. *J. Anal. Toxicol.* **32:** 31–36 (2008).
21. J.P. Langenberg, G.P. van der Schans, H.E.T Spruit, W.C. Kuijpers, R.H. Mars-Groenendijk, H.C.M van Dijk-Knijnenburg, H.C. Trap, H.P.M. van Helden, and H.P. Benschop. Toxicokinetics of sulfur mustard and its DNA-adducts in the hairless guinea pig. *Drug. Chem. Toxicol.* **21(suppl.1):** 131–147 (1998).
22. J.B. Morris. Nasal toxicology. In *Inhalation Toxicology,* 2nd ed., H. Salem and S.A. Katz, Eds. Taylor & Francis, Boca Raton, FL, 2006, pp 349–371.
23. B.J. Benton, J.M. McGuire, D.R. Sommerville, P.A. Dabisch, E.M. Jakubowski, Jr., K.L. Matson, R.J. Mioduszewski, and C.L. Crouse. Effects of whole-body VX vapor exposure on lethality in rats. *Inhal. Toxicol.* **18:** 1091–1099 (2006).